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# Coexistence of Several Novel Hantaviruses in Rodents Indigenous to North America

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Three genetically distinct members of the Hantavirus genus have been detected in Nevada rodents by RT-PCR and nucleotide sequence analysis. These include Sin Nombre (SN), El Moro Canyon (ELMC), and Prospect Hill (PH)-like viruses which are primarily associated with *Peromyscus maniculatus* (deer mouse), *Reithrodontomys megalotis* (western harvest mouse), and *Microtus* spp. (voles), respectively. Although this region of the United States is ecologically diverse, rodents infected with different hantaviruses appear to coexist in several different geographical and ecological zones. In two widely separated states, Nevada and North Dakota, PH-like viruses are present in three different species of vole. In addition, ELMC-like virus has been detected in both *R. megalotis* and *M. montanus* (mountain vole). SN virus is a cause of hantavirus pulmonary syndrome throughout much of the United States. SN virus RNA is found in 12.5% of *P. maniculatus* in Nevada and eastern California. Two lineages of SN virus coexist in this region and differ from SN viruses originally found in infected rodents in New Mexico, Arizona, and Colorado. These data show the complexity of hantavirus maintenance in rodents. Distinct hantaviruses or virus lineages can coexist either in different or the same rodent species and in either different or the same geographic or ecological zones. 

## INTRODUCTION

Members of the *Hantavirus* genus (family *Bunyaviridae*) are distributed world-wide, each associated with a specific rodent host (LeDuc, 1987; LeDuc *et al.*, 1992). The rodent hosts develop a persistent or chronic infection with no apparent disease symptoms (LeDuc, 1987; Lee and van der Groen, 1989). The Old World hantaviruses, Hantaan (HTN), Seoul (SEO), and Puumala (PUU) are causative agents of hemorrhagic fever with renal syndrome (HFRS), which ranges from a mild to serious human disease affecting primarily the kidneys (Chen and Yang, 1990; Childs *et al.*, 1991; LeDuc *et al.*, 1992). The 1993 outbreak in the southwestern United States of a previously unrecognized disease, now termed hantavirus pulmonary syndrome (HPS), lead to the identification of Sin Nombre virus (SN) (originally referred to as Four Corners or Muerto Canyon virus), a novel hantavirus, as the causative agent (Elliott *et al.*, 1994; Nichol *et al.*, 1993). Prior to the identification of SN virus the only known hantaviruses in the United States were Prospect Hill virus (PH) (Lee *et al.*, 1985; Tsai *et al.*, 1985; Yanagihara *et al.*, 1987) and SEO (Gott *et al.*, 1993; LeDuc *et al.*, 1992, 1984; Tsai *et al.*, 1985). Antibodies reactive with PH virus have been found in humans in the United States, but it is not known whether this virus infects humans (Yanagihara, 1990; Yanagihara *et al.*, 1984). PH virus is found in the indigenous North American meadow vole, *Microtus pennsylvanicus* (Lee *et al.*, 1985), while the rodent host for SEO virus is the Norwegian rat, *Rattus norvegicus* (Lee *et al.*, 1982) which has spread SEO virus to cities worldwide, particularly ports (Glass *et al.*, 1993). The rodent host for SN virus was determined to be the deer mouse, *Peromyscus maniculatus* (Childs *et al.*, 1994; Nichol *et al.*, 1993). Subsequent reports of novel HPS-associated hantaviruses in the New World include Black Creek Canal virus (BCC) in Florida, carried by the cotton rat, *Sigmodon hispidus* (Ravkov *et al.*, 1995; Rollin *et al.*, 1995), Bayou virus (BAY) from a human HPS case in Louisiana, with no rodent host yet identified (Khan *et al.*, 1995; Morzunov *et al.*, 1995), and Rhode Island virus (RI-1) from a human HPS case in the Eastern United States (Hjelle *et al.*, 1995), with the white-footed mouse, *Peromyscus leucopus*, the possible rodent host (Song *et al.*, 1994). Also recently reported, but with disease status in humans unknown, are El Moro Canyon hantavirus (ELMC) and Rio Segundo hantavirus (RS) (formerly referred to as harvest mouse hantaviruses HMV-1 and HMV-2), identified in *Reithrodontomys* species in the Southwestern United States (Hjelle *et al.*, 1994a), Mexico, and Costa Rica (Hjelle *et al.*, 1994b).

Hantaviruses possess a negative sense, single-stranded RNA genome which consists of three segments. The large segment (L) encodes the viral polymerase, the medium segment (M) encodes glycoproteins G1 and G2, and the small segment (S) encodes the nucleocapsid protein (Antic *et al.*, 1991a, 1991b; Arikawa *et al.*, 1990; Parrington *et al.*, 1991; Schmaljohn *et al.*, 1986, 1987; Spiropoulou *et al.*, 1994).

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Many isolated mountain ranges with varying habitats are found in Nevada. The ranges are often separated by dry alkaline plains which may serve as natural barriers to the migration of small rodents. Several human cases of HPS reported in Nevada and eastern California suggested that rodents in this region carry hantavirus. We were interested in seeing how the hantaviruses present in this diverse ecological area were related to the viruses detected elsewhere in the United States. We had recently sampled over 1800 rodents from 27 species in this region, and observed that *P. maniculatus, P. truei, R. megalotis, M. montanus,* and *Ammospermophilus leucurus* possess antibody against SN nucleocapsid antigen (Otteson *et al.,* 1995). As the nucleocapsid antigen is broadly cross-reactive with several New World hantaviruses (Ksiazek *et al.,* 1995), we wanted to identify the specific hantavirus types infecting the different rodent species.

## MATERIALS AND METHODS

### Rodents and serological screening

The majority of rodents analyzed in this study were obtained by the considerable efforts of several state agencies in Nevada, California, and North Dakota from 1993 to 1995. Procedures and results of serological screening have been published elsewhere (Otteson *et al.,* 1995).

### RNA extraction, RT-PCR amplification, and sequencing

To avoid RNA template or PCR product cross-contamination, RNA was extracted and purified in a biohazard containment hood in a biosafety level 3 (BSL3) facility, and RT-PCR reactions were performed in a biohazard containment hood in a separate laboratory. Total RNA was extracted from 10 mg of lung tissue or blood clot using an RNaid Plus kit (Bio 101, La Jolla, CA) according to the manufacturer's instructions. Several sets of primers with sequence similarity to published hantaviruses (PUU, PH, ELMC, SN, and BAY) were used in nested RT-PCR, performed as described previously (Nichol *et al.,* 1993). The products generated were separated on 1% agarose gels, and the fragment of interest was excised from the gel and purified using the Geneclean kit (Bio 101, La Jolla, CA) according to the manufacturer's instructions. The purified PCR product was then sequenced directly by the dideoxy primer extension method (Winship, 1989), using Sequenase (Amersham) followed by a Terminal deoxynucleotidyl Transferase chase step (DeBorde *et al.,* 1986) or dyedeoxy cycle sequencing (Applied Biosystems, CA).

### Oligonucleotide primers for PCR

Primers initially used for RT-PCR amplification of a 139-bp fragment (position 2803 to 2941) of the G2 coding region of the M segment of SN virus have been previously published (Nichol *et al.,* 1993). These primed poorly with one Nevada lineage of SN virus; new nested primer pairs were designed which would detect both Nevada lineages and the New Mexico, Arizona, and Colorado lineages. The sequences are: first round, 5′TGTGTGTTTGGAGACCCTGG3′ (M2662F) and 5′TC(A/G)ATAGATTGTGTATGCA3′ (M2999R); second round 5′ATGTCAACAAC(A/G)AGTGGGATG3′ (M2689F) and 5′CATGGGTTATCACTTAG(G/A)TC3′ (M2969R). When seropositive rodents were found in Nevada *Reithrodontomys* and *Microtus* species, which gave no M segment RT-PCR products, new primer pairs were designed from conserved regions between PH and BAY published sequences (Morzunov *et al.,* 1995; Parrington *et al.,* 1991). The primers used for amplification of Nevada PH-like samples were: first round, 5′TGAAACAGGGTGGGGCTGTAATCC3′ (M2340F) and 5′ACTCCGCAGGAACAAAAG3′ (M3687R); second round, 5′GCTACAATGCCAACATGTGAGTA3′ (M2755F) and 5′TCACATGCCTTTATTGAAGTTA3′ (M3092R). M segment primers used for amplification of North Dakota *Microtus* samples were the PUU/PH primers described previously (Nichol *et al.,* 1993).

Primer sequences to the S segment were designed from conserved sequence regions between PH, SN, and ELMC published sequences (Hjelle *et al.,* 1994a; Parrington and Kang, 1990; Spiropoulou *et al.,* 1994). The S segment primer sequences are: 5′GGAATGAGCACCCTCAAAGAAGTGCAAGACAAC3′ (S41F), 5′TGGACCpC(A/C)GATGA(C/T)GTTAACAA3′ (S143F), 5′GGGCAGCTGTGTCTGCATTG3′ (S185F), 5′ACATCAAGGACATT(T/C)CCATA3′ (S353R), 5′AT(A/G)GT(A/G)TT(C/T)CTCATATCCTG3′ (S1064R), 5′ATTATATCTTTAGTGGTTCTTGGTTAGAGATTTCCC3′ (S1319R). Because of the sequence variability among samples, several primer combinations were sometimes tried on each sample before a successful combination of primers was found. Some samples gave no RT-PCR products with certain segment primer pairs, and others gave very small amounts of product, indicating mismatched primers. The primer combinations used for amplification of the different virus types are listed in Table 1.

### Computer analysis

Sequence comparisons and alignments were performed on a DEC Alpha 7000 running OSF/1 UNIX, using GAP, PILEUP, and LINEUP programs from the Wisconsin Package version 8 (Genetics Computer Group, Madison, WI). Phylogenetic analysis was performed using the PAUP 3.1.1 program (David L. Swofford, Smithsonian Institute, Washington, DC) on a Macintosh PowerPC 6100. For both the S and M segments of the genome, a weighted, maximum parsimony, heuristic analysis was performed on the sequence data from RT-PCR products and several published sequences. The resulting unrooted trees were outgrouped to HTN and SEO sequences. To account for transversion and transition dis-

TABLE 1

First and Second Round PCR Primers Used to Amplify S and M Segment Sequences from Various Rodent Species

| Virus type | Primer sets | | Second round product size (bp) |
|---|---|---|---|
| | First round | Second round | |
| SN | S41F, S1319R | S143F, S353R | 211 |
| | M2662F, M2999R | M2689F, M2969R | 281 |
| PH | S143F, S1064R | S143F, S353R | 211 |
| | M2340F, M3687R | M2755F, M3092R | 338 |
| | M2671F, M3108R | M2770F, M3012R | 242 |
| ELMC | S41F, S1319R | S143F, S1064R | 922 |
| | S41F, S1319R | S185F, S1064R | 880 |
| | M2340F, M3687R | M2755F, M3092R | 338 |

equilibrium, the analysis was weighted 4:1 for transversions over transitions. Bootstrap confidence limits were obtained from 200 heuristic search replicates.

### Nucleotide sequence accession numbers

For the M segment comparison, the following published sequences were used: HTN virus, strain 76-118 (M14627, Y00386) (Schmaljohn *et al.,* 1987; Yoo and Kang, 1987); SEO virus, strain SR-11 (M34882) (Arikawa *et al.,* 1990); PUU virus, strains Sotkamo/V-2969/81 (X61034) (Vapalahti *et al.,* 1992), Udmurtia/894Cg/91 (Z21509) (Plyusnin *et al.,* 1994), Bashkiria/CG18-20/84 (M29979) (Giebel *et al.,* 1989); PH virus, strain PHV-1 (X55129) (Parrington *et al.,* 1991); BAY virus (L36930) (Morzunov *et al.,* 1995); SN virus, NM H10 (L25783), NM R17 (L27786), NM R27 (L27780), NM R31 (L27791), AZ R19 (L27765), CA R36 (L27770), CA H19 (L27769), and CO R1 (L27772) (Spiropoulou *et al.,* 1994); Four Corners hantavirus, isolates CC74 (L33684), CC107 (L33474) (Li *et al.,* 1995), strain SWC1 (U10889) (Nerurkar *et al.,* 1994).

Published sequences used for the S segment comparisons were: HTN virus, strain 76-118 (M14626) (Schmaljohn *et al.,* 1986); SEO virus, strain SR-11 (M34881) (Arikawa *et al.,* 1990); PUU virus, strains Sotkamo/V-2969/81 (X61035) (Vapalahti *et al.,* 1992), Udmurtia/894Cg/91 (Z21497) (Plyusnin *et al.,* 1994), Bashkiria/CG18-20/84 (M32750) (Stohwasser *et al.,* 1990); PH virus, strain PHV-1 (M34011, X55128) (Parrington and Kang, 1990); TUL virus, strains 53Ma/87 (Z30942), 76Ma/87 (Z30942), 249Mr/87 (Z30944) (Plyusnin *et al.,* 1994); BCC virus (L39949) (Ravkov *et al.,* 1995); BAY virus (L36929) (Morzunov *et al.,* 1995); ELMC Hantavirus, strains RM-45 (U11425), RMNK-51 (U11430), RM-97 (U11427), and NMNK-164 (U11429) (Hjelle *et al.,* 1994a); SN virus, strain NM H10 (L24784) (Spiropoulou *et al.,* 1994); Four Corners hantavirus, isolates CC74 (L33816), CC107 (L33683) (Li *et al.,* 1995).

Accession numbers for the newly reported M segment sequences are: NV LY-R2312 (U33240), ND R737 (U33247), ND R731 (U33245), ND R789 (U33251), ND R812 (U33254), ND R742 (U33256), NV WA-R2025 (U33262), NV CC-R1645 (U33225), NV WA-R211 (U33262), CA BU-R615 (U33222), CA BU-R986 (U33224), CA BU-R982 (U33223), CA BU-R611 (U33221), CA BU-R608 (U33219), CA NE-H395 (U33445), NV WA-R199 (U33258), NV CC-R3 (U33227), NV CL-R304 (U33229), NV LY-R2302 (U33239), NV HU-R954 (U33238), NV WA-R1671 (U33257), NV LY-R792 (U33242), NV WA-H194 (U33264), CA MO-H295 (U33244), NV CL-R313 (U33230), NV CL-R341 (U33231), NV EU-R451 (U33234), and NV CL-H894 (U33233). Accession numbers for the newly reported S segment sequences are: ND R812 (U33255), ND R743 (U33250), ND R731 (U33246), ND R737 (U33248), ND R742 (U33249), ND R789 (U33252), ND R810 (U33253), NV LY-R2312 (U33241), NV CC-R1645 (U33226), NV WA-R2025 (U33261), CA BU-R608 (U33220), NV WA-R211 (U33263), NV LY-R792 (U33243), NV EU-R451 (U33235), NV CL-R341 (U33232), NV EU-578 (U33237), NV WA-R199 (U33259), and NV R-CC3 (U33228).

## RESULTS

### Comparison of RT-PCR and ELISA results

We were particularly interested in knowing whether blood was sufficient for the detection of virus sequence or if it was important to test other tissues. To conduct the long term field studies planned by this laboratory to follow the course of viral infection in natural rodent populations, the animals must be released unharmed after sampling and resampled at later times. To determine whether there was RNA with hantavirus-like nucleotide sequences present in both the blood clots and lung tissue of the animals, RNA was extracted from blood clot and lung tissue, and RT-PCR was performed.

Blood clots from 105 rodents were tested by RT-PCR and compared to ELISA results (Table 2). Of ELISA positive *M. montanus, R. megalotis,* and *P. truei,* 25, 33.3,

TABLE 2

Comparison of RT-PCR of Blood Clot and Lung Tissue with ELISA for Various Rodent Species

Comparison of ELISA and RT-PCR of blood clot

| Species | ELISA positive | RT-PCR positive | ELISA negative | RT-PCR positive |
|---|---|---|---|---|
| *P. maniculatus* | 51 | 37 (72.5%) | 40 | 2 (5.0%) |
| *P. truei* | 4 | 1 (25.0%) | | not tested |
| *M. montanus* | 7 | 1 (14.3%) | | not tested |
| *R. megalotis* | 3 | 1 (33.3%) | | not tested |

Comparison of ELISA and RT-PCR of lung tissue

| Species | ELISA positive | RT-PCR positive | ELISA negative | RT-PCR positive |
|---|---|---|---|---|
| *P. maniculatus* | 23 | 21 (91.3%) | 61 | 4 (6.6%) |
| *M. montanus* | 2 | 2 (100%) | | not tested |

and 14.3%, respectively, were also positive for hantavirus nucleotide sequence by RT-PCR. The *M. montanus* and *R. megalotis* samples were negative by RT-PCR when using the nested primer sets designed for SN virus, but positives were detected when primer sets with similarity to the published PH or ELMC sequences were used. Of the ELISA positive *P. maniculatus,* 72.5% were also positive for SN virus nucleotide sequence by RT-PCR, indicating that virus nucleotide sequence could be identified even in the presence of circulating antibodies in a large percentage of the *P. maniculatus.* No RT-PCR product was obtained from *A. leucurus.*

Lung tissue from 86 rodents was tested by RT-PCR and compared to ELISA results. Of the ELISA-positive animals, virus nucleotide sequence could be found in the lungs of 100% of *M. montanus* and 91.3% *P. maniculatus,* a significantly higher percentage than found in blood clots, indicating that the virus persists in the lungs even after it is cleared from the blood. No lung tissue was available for *R. megalotis, P. truei,* or *A. leucurus.*

Of the ELISA-negative *P. maniculatus,* 5% were positive by blood clot RT-PCR and 6.7% were positive by lung RT-PCR. These may represent recently infected animals which have not yet developed antibodies.

### Coexistence of genetically divergent hantaviruses

A 139-bp fragment from the G2 coding region of the M segment (position 2803 to 2941) and a 133-bp fragment from the coding region of the S segment (position 190 to 322) were amplified and sequenced to determine whether the viral nucleic acids detected in rodents represented SN virus or other hantaviruses. These nucleotide sequences were compared by phylogenetic analysis to published hantavirus sequences. It was impractical to compare full-length gene segment sequences when looking at a large number of samples; however, several studies have shown that the phylogenetic trees obtained from small gene fragment sequences are congruent with the trees obtained from an entire gene sequence although there may be minor rearrangements in the terminal branches. (Nichol *et al.,* 1993; Spiropoulou *et al.,* 1994; Liang *et al.,* 1994; Xiao *et al.,* 1994; Morzunov *et al.,* 1995; Ravkov *et al.,* 1995; Khan *et al.,* 1995; Hjelle *et al.,* 1995).

Figure 1 shows the one M segment tree obtained from a detailed phylogenetic analysis of hantavirus-infected rodents and human HPS cases. While several of the samples are from the same ecological zone in Nevada, various geographic regions and wide ecological diversity are also represented by the Nevada, eastern California, North Dakota, and published sequences. The five North Dakota *Microtus* (ND R731, ND R737, ND R742, ND R789, and ND R812) and two Nevada *Microtus* (NV LY-R2312 and NV LY-R2313) are more closely related to PH than any of the other known groups. Sequence divergence between PH and the North Dakota *Microtus* samples

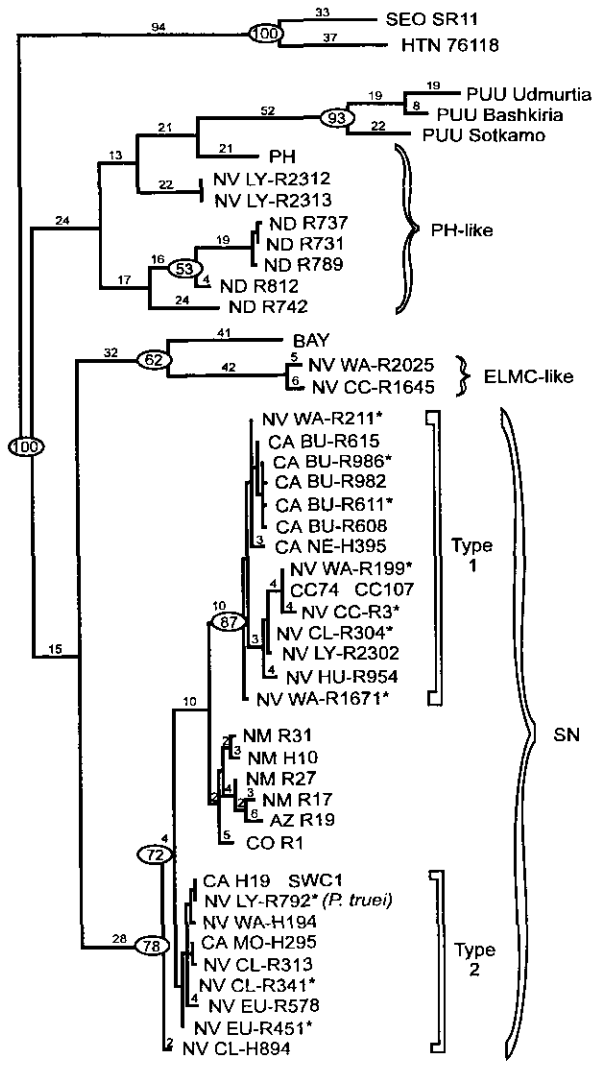


FIG. 1. Phylogenetic relationship of Nevada, California, and North Dakota hantaviruses to previously characterized hantaviruses. Phylogenetic analysis of 139 nucleotide region from the M segment (position 2803 to 2941) carried out by the maximum parsimony method using PAUP software. Horizontal line lengths are proportional to nucleotide step differences (indicated above each branch). Because the analysis was weighted 4:1 for transversions over transitions, nucleotide steps are not the number of nucleotide differences. For clarity, labels have been omitted on branch lengths with a value of 1, and bootstrap confidence limits have been omitted for the terminal taxa. Bootstrap confidence limits greater than 50% are circled at each major branch. Vertical lines are for graphic representation only. Abbreviations for states are: Arizona, AZ; California, CA; Colorado, CO; Nevada, NV; New Mexico, NM; North Dakota, ND. Abbreviations for counties are: Butte, BU; Carson City, CC; Clark, CL; Elko, EL; Eureka, EU; Humboldt, HU; Lyon, LY; Nevada, NE; Washoe, WA. R indicates rodent; H indicates human; * indicates several samples with the same sequence: NV WA-R211 represents NV CC-R1635; CA BU-R986 represents CA BU samples R2198, RR2201, R2206, R2208, R2381, R2714; CA BU-R611 represents CA BU-R616; NV WA-R199 represents NV HU samples R885, R886, R893, R900, R910 and NV LY samples R724, R725, R726, R730, R742, R763; NV CC-R3 represents NV CC samples R6, R17; NV CL-R304 represents NV CL-R306 and NV EU-R693; NV WA-R1671 represents NV WA samples R1672, R1689, R1701; NV LY-R792 represents NV LY samples R758, R786, R2296 and CA MO samples R2628, R2637, R2648; NV CL-R341 represents NV EL-R1714; NV EU-R451 represents NV HU-R901, NV WA-R1657, NV EU samples R452, R455, R576 and NV LY samples R735, R760, R762.

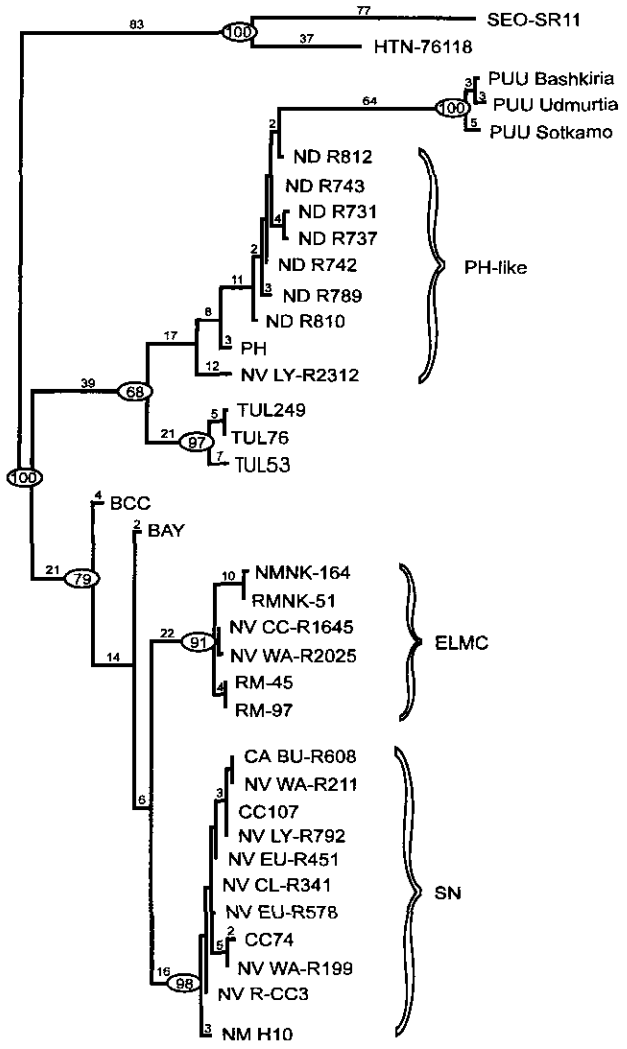


FIG. 2. Phylogenetic relationship of Nevada, California, and North Dakota hantaviruses to previously characterized hantaviruses. Phylogenetic analysis of 133 nucleotide region from the S segment (position 190 to 322) carried out by the maximum parsimony method, using PAUP software.

ranges from 19.4 to 22.3% in the 139 nucleotide M segment fragment analyzed for this study while divergence between PH and the Nevada *Microtus* samples is 20.1%. PH virus infection has previously been reported to occur in the meadow vole, *M. pennsylvanicus,* in Maryland, Wisconsin, and Minnesota (Burek *et al.,* 1994; Lee *et al.,* 1985). From the analysis presented here, it appears that the PH-like group of hantaviruses is capable of infecting three species of *Microtus,* in geographical regions spanning the United States and from diverse ecological zones. No TUL virus M segment sequence was available for comparison with the North Dakota *Microtus,* but an S segment analysis enabled us to relate TUL virus to PH virus and the Nevada and North Dakota *Microtus* samples. Figure 2 shows one of five equally parsimonious trees obtained from phylogenetic analysis of S segment nucleotide sequence data. The five trees arose from minor rearrangements of the terminal taxa of the ELMC branch. TUL virus has been reported as a distinct novel member of the genus *Hantavirus,* related to PH virus (Plyusnin *et al.,* 1994). The analysis presented here shows that TUL and all the PH-like viruses form a distinct clade, with sequence divergence from PH virus ranging from 18.3 to 19.8% for the S segment sequence analyzed. Nucleotide sequence divergence of 9.8% is seen between PH virus and the PH-like virus detected in *Microtus* from Nevada, and 6.8 to 10.5% between PH virus and North Dakota *Microtus* samples, showing a closer relationship between viruses from the New World *Microtus* spp. Since the sequences were obtained by directly sequencing the RT-PCR product (not derived by cloning single copies of the RT-PCR product), they represent the average sequence present, and the differences observed cannot be attributed to reverse transcriptase or taq polymerase errors which may occur during the RT-PCR amplification procedure.

ELMC-like virus was also detected in one *R. megalotis* (NV CC-R1645) and in one *M. montanus* (NV WA-R2025) in Nevada. No published ELMC virus sequence was available for the M segment, but it can be seen from the S segment analysis (Fig. 2) that the two Nevada samples are clearly members of the ELMC virus group. The published sequences for ELMC were obtained from rodents in eastern Arizona (NMNK-164 and RMNK-51) and California (RM-45 and RM-97), showing that this hantavirus also has a wide geographical distribution (Hjelle *et al.,* 1994a). Although *R. megalotis* is considered the primary rodent reservoir for ELMC, the virus was also found in a woodrat (*Neotoma mexicanus*) (Hjelle *et al.,* 1994a), and we have identified it in *M. montanus.* The divergence seen between the published ELMC nucleotide sequences and those from Nevada, ranges from 3.8 to 5.3%.

## Two lineages of SN viruses coexist in Nevada

The relationship of Nevada and eastern California SN virus nucleotide sequences to other reported SN virus sequences was examined. The complete M and S sequence of two eastern California (Mono county) isolates, designated CC74 and CC107, has been reported (Li *et al.,* 1995), as well as the partial M segment sequence of two Mono county samples; CA H19, a human HPS case, and CA R36 (Spiropoulou *et al.,* 1994), the original rodent sample from which CC107 was isolated (Schmaljohn *et al.,* 1994). Also the virus sequence obtained from a 10-year-old *Peromyscus* sample from Sweetwater Canyon (SWC1), in the same geographical region, has been published (Nerurkar *et al.,* 1994). We report partial M and S nucleotide sequences which are in agreement with those already published. However, we have compared virus nucleotide sequences obtained from four human cases of HPS, and 58 rodents from diverse ecological zones throughout the state of Nevada and three regions of eastern California (Mono, Nevada, and Butte counties), in order to examine virus diversity within the SN group. Two

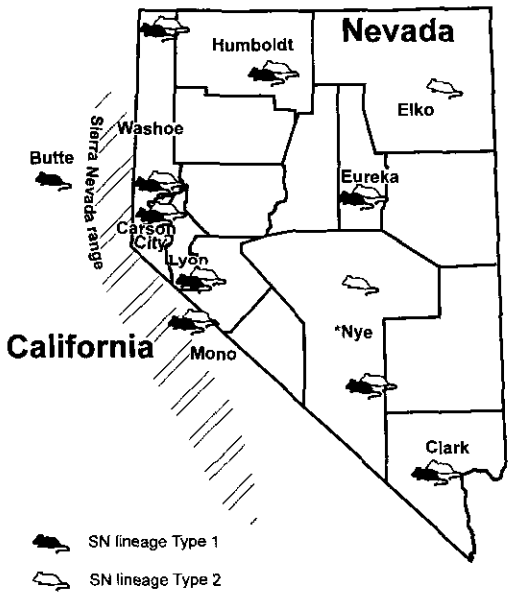


FIG. 3. Distribution of 2 SN virus lineages within Nevada and California. *Nye county, data not shown.

main findings have emerged from phylogenetic analysis of a 139-bp nucleotide fragment of the viral M segment (Fig. 1). The Nevada and eastern California samples fall into two lineages, and these are distinct from the virus lineages present in New Mexico, Arizona, and Colorado areas (Spiropoulou *et al.*, 1994). The two lineages (Type 1 and Type 2) are supported by bootstrap confidence limits of 87 and 72%. The maximum nucleotide identity between Types 1 and 2 (NV WA-R211 compared with NV LY-R792) at 87.1%, is less than that between either of the types and the New Mexico samples. Type 1 (NV WA-R211) has 92.1% identity to NM R31, and Type 2 (NV LY-R792) has 90.6% identity to NM R31. These two lineages are found coexisting in Nevada *Peromyscus* populations in Carson City, Clark, Eureka, Humboldt, Lyon, and Washoe counties and in Mono county, California. These counties include much of Nevada. Figure 3 shows the distribution of the 2 lineages in Nevada and eastern California. Interestingly, we have found only the Type 1 lineage in Butte county, California. No SN variants of the type found in the New Mexico, Arizona, and Colorado areas were detected in Nevada or eastern California. M and S segment nucleotide sequence obtained for an ELISA positive *P. truei* (NV LY-R792) was identical to SN virus Type 2 found in *P. maniculatus* trapped in the same location.

## DISCUSSION

### PH-like virus found in diverse geographical regions of the United States

While the primary rodent reservoir for PH virus is *M. pennsylvanicus*, we have also identified PH virus-like nucleotide sequence in two *M. montanus* in Nevada and seven *Microtus* in North Dakota (three *M. ochrogaster* and four *M. pennsylvanicus*). The recently reported TUL virus which is also related to PH virus was found in two different European *Microtus* species, and it was suggested that both species were the rodent reservoir for this virus (Plyusnin *et al.*, 1994). There have been no reports of a PH-like virus in any genus other than *Microtus; however, there may be considerable flexibility in the* ability of PH-like viruses to infect different species of the *Microtus* genus. The phylogenetic analysis presented here suggests there is no clear correlation that virus speciation occurs within specific *Microtus* species; however, the coevolution of virus with rodent host, which is characteristic of the *Hantavirus* genus, does appear to occur, at least to the *Microtus* genus level. An alternate explanation of these data may be that secondary infection (spill over of virus infection from the coevolving rodent host into other rodent species) occurs readily with the PH-like viruses. Analysis of a larger number of *Microtus* should enable that distinction to be made.

### Three hantaviruses coexist in Nevada

It is particularly interesting that three hantaviruses coexist in the rodent population in Nevada. PH-like virus RNA is found in *M. montanus*, ELMC-like virus in *R. megalotis* and *M. montanus*, and SN virus in *P. maniculatus* and *P. truei*. The habitats of these rodents overlap in many areas, but the specificity of virus with rodent host appears to be maintained, at least to the rodent genus level with PH and SN viruses. RT-PCR positive *Peromyscus* and *Microtus* were captured in the same river bank ecological zone in Lyon county, yet PH-like virus RNA was detected only in the genus *Microtus* and SN virus RNA only in the genus *Peromyscus*.

### Two lineages of SN virus coexist in Nevada and eastern California

When we began this study, it was to examine the phylogenetic relationships of SN virus variants from the perspective of the natural barriers to rodent movement afforded by Nevada's unique geographical features. In the basin and range topography of Nevada, there are over 100 north–south mountain ranges up to 13,000 ft in elevation, separated by flat valleys up to 40 miles wide. The valleys are lower in elevation by several thousand feet, often with large areas of dry alkali flats in which the summer temperatures are extremely high. We thought it likely that there would be very little movement of rodents between the ranges and that if virus had been present in the rodent population for an extended time, we may find genetic variants specific to each mountain range. This genetic pattern did not emerge. Based on M segment sequence we found two lineages of SN virus, and at all locations in Nevada where we have identified more than three positive rodents, both lineages are present. Over the 139 nucleotide sequence examined, there is

greater than 95.0% identity within Type 1 lineage and greater than 97.1% identity within Type 2, while between the two lineages there is less than 87.1% identity. The lineages are distinctly different from those found in New Mexico, Arizona, and Colorado, indicating that geographical location does play some role in the distribution of genetic variants.

The basin and range uplift and extension of Nevada's Great Basin began to occur about 17 million years ago and continues today. With the onset of the ice ages, about 3 million years ago, lakes filled many of the valleys and the Great Basin became an inland sea (Fiero, 1986). The last pulse of the ice ages ended about 12,000 years ago, and the lakes began to recede (Moore, 1969) leaving the dry valleys we see today. During the ice age period, the lake levels fluctuated as glaciers repeatedly formed and retreated. While temporarily isolating the mountain peaks, the vast amount of water in the Great Basin ameliorated the climatic extremes between range and valley. A cooler, savannah-like biome, with abundant water, may have encouraged the spread of rodents across the Great Basin, and rodent populations may have been interconnected at least until the last few thousand years. It is possible that *P. maniculatus* populations are still interconnected today. *P. maniculatus* is a generalist, occupying ecological zones which range from high mountains with deep winter snow to dry sagebrush flats. The coexistence of the two SN lineages in Nevada may also be maintained by the genetic stability of the virus. Once the two lineages were established, if environmental conditions in the rodents (both cellular, and broader influences) remained constant, then minor mutants of the virus, with no fitness advantage, would not replace the dominant lineage.

It is of interest that only one lineage has been found in 12 samples from Butte county in California, which is located on the western slope of the Sierra Nevada range, the highest range in the continental United States. In Mono county, which encompasses the eastern face of the Sierra Nevada, both lineages are present. The Sierra Nevada forms the western boundary of the Great Basin and may have been an effective barrier to rodent movement during the ice ages, when it was extensively glaciated. However, it seems likely that interconnected populations of *P. maniculatus* occur throughout the Sierra Nevada today, as large *P. maniculatus* populations are found at the elevation of several mountain passes.

### Frequency of hantavirus infection of the rodent population of Nevada

Results of serological testing in Nevada have been reported elsewhere (Otteson *et al.*, 1995), but in this study we have related the specific hantavirus type infecting various rodents, (determined by RT-PCR, sequencing and phylogenetic analysis) to the frequency of seropositivity in the rodents. Rodent sera were tested by ELISA, using SN virus (NM H10 variant) recombinant nucleocapsid protein (Feldmann *et al.*, 1993). Antibodies to several different hantaviruses are known to cross-react with this antigen (Ksiazek *et al.*, 1995). SN virus detected in New Mexico, Arizona, and Colorado has been found in species other than *P. maniculatus*, but of 1687 rodents from 29 species collected in that region, 48% were *P. maniculatus* (Childs *et al.*, 1994), showing this rodent to be by far the most prevalent species. Similar findings were reported for the Nevada and eastern California region. Of 1832 rodents from 28 species collected, 51% were *P. maniculatus*, and of the 938 *P. maniculatus* tested by ELISA, 12.5% were positive for the presence of antibody (Otteson *et al.*, 1995).

The average percentage of antibody positive rodents at the time of the 1993 human epidemic of SN virus in the New Mexico, Arizona, and Colorado area was 30%, with a spill over into 10 other rodent species (Childs *et al.*, 1994). In contrast, the overall frequency of positive *P. maniculatus* in Nevada is 12.5%, with a spill over to 1 other rodent species (*P. truei*). It is possible that the differences in frequency of antibody positive *P. maniculatus* between the two study areas reflects the difference between the enzootic and epizootic incidence of virus infection. Spill over into other species may not occur unless both the incidence of virus infection in *P. maniculatus* and the rodent density is high. The 12.5% hantavirus seropositivity found in Nevada rodents is an average of foci of positive animals ranging from 0 to 51% (data not shown). The 30% seropositivity reported in 1993 was also an average figure of antibody prevalence which varied from 9.5 to 38.6% in New Mexico, from 27.1 to 35.4% in Arizona, and from 13.3 to 51.3% in Colorado. In any foci where 12 or more *P. maniculatus* were examined, ELISA positive rodents were found (Childs *et al.*, 1994). In contrast, in several eastern California and Nevada foci, no virus was detected (Otteson *et al.*, 1995). Since the maximum degree of antibody prevalence in these regions is similar, the lower overall percentage found in Nevada must result from considerably more foci with no detectable virus. There has not been a human HPS outbreak of epidemic proportions in Nevada and eastern California. There have been 10 cases reported between March 1993 and April 1995, with two deaths. It may be possible that the average frequency of antibody positive *P. maniculatus* can be used as a guide in anticipating a human epidemic of SN virus.

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